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**Surface Display of Proteins on Bacteriophage  
 $\lambda$  Heads**

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## Surface Display of Proteins on Bacteriophage $\lambda$ Heads

Y. Gi Mikawa<sup>1</sup>, Ichi N. Maruyama<sup>1\*</sup> and Sydney Brenner<sup>2</sup>

<sup>1</sup>Department of Cell Biology  
The Scripps Research  
Institute, 10666 North Torrey  
Pines Road, La Jolla  
CA 92037, USA

<sup>2</sup>Department of Medicine  
University of Cambridge  
Hills Road, Cambridge  
CB2 2QQ, England

We have developed plasmid and phage vectors for the display of foreign proteins on the surface of bacteriophage  $\lambda$  capsid by modifying the *D* gene which encodes the major head protein gpD. The vectors have multiple cloning sites, and permit colour selection and conditional chain termination for recombinants. Displayed proteins can be fused to either the N or C terminus of gpD by a peptide linker. The conditional chain termination scheme, via a host *Escherichia coli* suppressor activity, allows the fusion and assembly of homomultimeric proteins as well as control of the number of fusion proteins per phage particle. We have successfully displayed  $\beta$ -lactamase, IgG-binding domains of the *Staphylococcus aureus* protein A, and  $\beta$ -galactosidase by cloning the genes into the vector. The constructs express functionally active proteins fused to gpD that assemble into phage particles. These results suggest that gpD may be fused to many other peptides and proteins at their N or C terminus and the fusion products may be accessible on the surface of bacteriophage  $\lambda$  particles.

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\*Corresponding author

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### Introduction

The fusion phage system introduced by Smith (1985) displays foreign peptides or proteins on the surface of filamentous bacteriophage particles *via* fusion to the phage coat proteins. This has been widely used to select particular clones from a large ensemble by construction of libraries of variant peptides, which can be searched for *in vitro* by affinity selection (Wells & Lowman, 1992). Other surface expression schemes have been devised for bacterial cells (Lu *et al.*, 1995) and for animal viruses (Kasahara *et al.*, 1994). However, all of these, including filamentous phage, require the fusion to be translocated across the plasma membrane, and there are many cytoplasmic proteins that cannot be extruded as fusion proteins. We previously reported a bacteriophage  $\lambda$  vector,  $\lambda$ foo, for the display of foreign proteins fused to the V protein, a major protein of the phage tail, which is more appropriate for the display of proteins that undergo folding in the cytoplasm (Maruyama *et al.*, 1994). The vector has been successfully used for the display of homo-multimeric proteins such as

$\beta$ -galactosidase and a plant lectin, BPA. Although these fusion constructs have been purified efficiently by panning on a microtiter plate coated with specific antibodies, the limitations of this system include the low expression level of fusion proteins, a few molecules per phage particle, and the low yields of phage after affinity purification, approximately 1%. In order to increase both the incorporation levels of fusion proteins and, hence, the recovery of fusion phage, we have further explored another  $\lambda$  phage coat protein, D protein, as the fusion partner of foreign proteins.

The D protein (gpD) has a molecular mass of 11 kDa and is essential for the stable phage head morphogenesis with wild-type genomes, although phages with less than 0.82 of the wild-type length can still be assembled in the absence of gpD. Structural analysis using cryo-electron microscopy has shown that gpD is assembled as trimers and appears as prominent protrusions on the capsid surface (Dokland & Murialdo, 1993). The conditional requirement for gpD in the phage capsid assembly and its location on the head surface have suggested that foreign proteins may be fused to gpD without interfering with phage assembly and the displayed proteins may be accessible for binding. During the course of this work, Sternberg and Hoess (1995) have reported a similar fusion system to the N terminus of gpD. Our system,

Abbreviations used: BSA, bovine serum albumin; gpD and gpV,  $\lambda$  phage proteins encoded by gene *D* and *V*, respectively; PCR, polymerase chain reaction; pfu, plaque-forming units.

described here, uses a conventional fusion scheme to either the N or C terminus of gpD and allows the assembly of multimer proteins into phage heads. The vectors have been designed to encode a long linker sequence consisting of proline and threonine repeats, an endopeptidase recognition sequence between gpD and foreign fusion proteins, and the *lacZ'* gene. The linker is to promote independent folding of the two domains, gpD and foreign proteins. The endopeptidase cleavage site allows infectious phage particles to be released from solid matrices under mild conditions. The *lacZ'* gene permits colour selection for recombinants in library construction.

## Results

### Modification of the *D* gene

In order to introduce a restriction site at the 5' end of the *D* gene, site-directed mutagenesis was carried out by the polymerase chain reaction (PCR) using  $\lambda$ 1685 DNA as a template, an oligonucleotide primer encoding a *Kpn*I site between the second and third codons, and a reverse primer encoding an *Eag*I site after the termination codon of the gene. The PCR product was cloned into pACYC184, resulting in p $\lambda$ D1 (for details, refer to Materials and Methods and Figure 1), in which glycine and threonine were inserted between the second and third amino acid residues of gpD. To test the activity of the modified gpD, an amber mutant of the *D* gene,  $\lambda$ F7 phage, was grown on an *Escherichia coli* host harbouring p $\lambda$ D1 and examined for EDTA sensitivity (for plasmid, phage and bacterial strains, see Table 1). In the absence of an *E. coli* amber suppressor,  $\lambda$ F7 with an immature head lacking gpD is produced and is extremely sensitive to EDTA. In contrast,  $\lambda$ F7 with gpD is resistant to EDTA treatment (Sternberg & Weisberg, 1977; Imber *et al.*, 1980).  $\lambda$ F7 grown on EQ166, a suppressor-negative strain, was sensitive to EDTA treatment, while  $\lambda$ F7 grown on EQ166 harbouring p $\lambda$ D1 showed weak EDTA resistance (Table 2). Although the activity of the modified gpD was very weak, this result indicated that gpD encoded by p $\lambda$ D1 could be supplied *in trans* and incorporated into phage particles. In order to raise the incorporation level of the modified gpD, we constructed a plasmid, p $\lambda$ D, using a high copy number plasmid vector, pBluescript II KS+ (pBSC). As shown in Table 2,  $\lambda$ F7 grown on EQ166

harbouring p $\lambda$ D showed almost complete resistance to EDTA, which is equivalent to that of phage grown on Q358, a suppressor-positive strain.

Next, we examined the incorporation of gpD fusion products into phage particles by constructing plasmids, p $\lambda$ AD and p $\lambda$ LD, which encode chimaeric proteins, *Staphylococcus aureus* protein A and  $\beta$ -lactamase fused to the N terminus of gpD, respectively (Figure 1). These fusion proteins also made  $\lambda$ F7 resistant to EDTA (Table 2), indicating that the fusion proteins could assemble onto  $\lambda$ F7 heads. The fusion proteins expressed from a high copy number plasmid, p $\lambda$ AD, provided the phage with greater resistance than those from a low copy number plasmid, p $\lambda$ LD. This result indicated again that the expression levels were critical for the efficient incorporation of the fusion gpD into phage heads. Phage containing the fusion protein produced by p $\lambda$ AD was more sensitive to EDTA than that containing unfused gpD produced by p $\lambda$ D, suggesting either that the heads with the fusion proteins were not completely stable against EDTA or that the incorporation of the fusions was not as efficient as the unfused gpD.

### Activity of proteins displayed on phage particles

To verify  $\beta$ -lactamase activity on phage particles,  $\lambda$ F7 grown on bacteria containing p $\lambda$ LD were fractionated through a CsCl density-gradient and  $\beta$ -lactamase activity was assayed with Nitrocefin (O'Callaghan *et al.*, 1972) as a substrate (Figure 2).  $\beta$ -Lactamase activity co-migrated with phage particles through the gradient, indicating that active  $\beta$ -lactamase was successfully incorporated into phage particles. The activity of the IgG-binding domains of the protein A molecule fused to gpD was verified by adsorbing  $\lambda$ F7 prepared from EQ166 harbouring p $\lambda$ AD to a microtiter plate coated with bovine serum albumin (BSA) after mixing the phage with rabbit anti-BSA antibody (Figure 3). The phage bound to the plate in an antibody concentration-dependent fashion but not to the plate without coated BSA. The results showed that the protein A domains on phage heads retained their IgG-binding activity. When grown on *E. coli* carrying p $\lambda$ AD, the  $\lambda$ 2001 phage (Karn *et al.*, 1984) with the wild-type *D* gene also bound to the plate as efficiently as did  $\lambda$ F7 (Figure 3), confirming that  $\lambda$ 2001 captured the fusion proteins on its capsid even in the presence of the wild-type gpD.

**Figure 1.** Construction of vectors. (a) Flow-chart of vector construction. A map of the left arm of  $\lambda$  phage is shown at the top. The following abbreviations are used; MCS, multiple cloning sites; pBSC, pBluescript II KS+;  $\Delta$ , deletion; c, collagenase recognition sequence; f, factor Xa recognition sequence; PA, protein A; AI, *Apa*I; ALI, *Apa*LI; Ba, *Bam*HI; EI, *Eag*I; RI, *Eco*RI; HIII, *Hind*III; KI, *Kpn*I; NI, *Nhe*I; PI, *Pst*I; PII, *Pvu*II; SI, *Sma*I; SFI, *Sfi*I. (b) The sequence between the second and third codons of the *D* gene on p $\lambda$ D5 and  $\lambda$ fooDn is shown. The collagenase and factor Xa recognition sequences, and the unique restriction sites are indicated. (c) The sequence of the fusion junction in p $\lambda$ D3 and  $\lambda$ fooDc. The sequences from the last codon, encoding 110th valine, of the *D* gene to *Eco*RI site in MCS are shown. The collagenase recognition sequence and the unique restriction sites are underlined. The ribosome-binding site of the *lacZ'* gene and the initiation codon methionine are indicated.



(c)

**NheI**

5' GTTATGCTTAGCAGCTGGCCGTATTGGGCCACTCGGACCCCGAACCTCCCACCCGACTCCCACCCCGAACCCCGACCCGACT  
V amb A S S W E V G F T P T P T T C T P T P T P T P T P T P T P T P T P T P T P T P T P T P T P T P T

**D110 collagenase HindIII**

CAGACCGTTGGGCCAATTGTCACAGAAGAACTATGACCATGATTACCGCAAGCTGCATGCCTGCAGGTCGACTCTAGAG  
P T V G P I V T Q E T A M T M I T P S L H A C R S T L E

**BamHI SacI EcoRI**

GATCCCCCGGTACCGAGCTGAATTCGGCACTGGCC  
D P R V P S S N S A L A

**Figure 1.**

Table 1. Plasmid, phage and bacterial strains

| Strains                  | Relevant properties                                       | Source/Reference                   |
|--------------------------|---|------------------------------------|
| <b>A. Plasmid</b>        |   |                                    |
| p $\lambda$ D            | Wild-type <i>D</i> gene in pBSC                           | This work                          |
| p $\lambda$ D1           | Wild-type <i>D</i> gene in pACYC184                       | This work                          |
| p $\lambda$ Do           | $\lambda$ DNA in pUM15                                    | This work                          |
| p $\lambda$ Ds           | <i>Sfi</i> I site in p $\lambda$ Do                       | This work                          |
| p $\lambda$ Dne          | <i>Nhe</i> I and <i>Eag</i> I sites in p $\lambda$ Do     | This work                          |
| p $\lambda$ Da           | MCS* in p $\lambda$ Ds                                    | This work                          |
| p $\lambda$ Db           | Linker sequence in p $\lambda$ Da                         | This work                          |
| p $\lambda$ D $\alpha$   | Linker sequence in p $\lambda$ Da                         | This work                          |
| p $\lambda$ D $\beta$    | Linker and <i>lacZ'</i> in p $\lambda$ Dne                | This work                          |
| p $\lambda$ D5           | Vector for fusion to N terminus of gpD                    | This work                          |
| p $\lambda$ D3           | Vector for fusion to C terminus of gpD                    | This work                          |
| p $\lambda$ LD           | $\phi$ ( $\beta$ -Lactamase-gpD) <sup>b</sup> in pACYC184 | This work                          |
| p $\lambda$ AD           | $\phi$ ( <i>S. aureus</i> protein A-gpD) in pBSC          | This work                          |
| p $\lambda$ DA           | $\phi$ (gpD-protein A) in pBSC                            | This work                          |
| pACYC184                 | Low copy number plasmid                                   | Chang <i>et al.</i> (1978)         |
| pBSC                     | pBluescript II KS+, high copy number plasmid              | STRATAGENE                         |
| pMC1871                  | Construct containing <i>E. coli lacZ</i>                  | Casadaban <i>et al.</i> (1983)     |
| pRIT5                    | Construct containing <i>S. aureus</i> protein A gene      | Nilsson <i>et al.</i> (1985)       |
| pUC18                    | Construct encoding $\beta$ -lactamase                     | Yanisch-Peron <i>et al.</i> (1985) |
| pUM13                    | Derivative of pBSC  | Maruyama & Brenner (1992)          |
| pUM15                    | Derivative of pUM13                                       | I. Maruyama                        |
| <b>B. Phage</b>          |   |                                    |
| $\lambda$ 1685           | Wild-type gpD   | Maruyama <i>et al.</i> (1994)      |
| $\lambda$ 2001           | Wild-type gpD   | Karn <i>et al.</i> (1984)          |
| $\lambda$ F7             | <i>imm21 cIts Dam15</i>                                   | S. Brenner                         |
| $\lambda$ $\beta$ gal    | $\phi$ (gpV- $\beta$ -galactosidase)                      | Maruyama <i>et al.</i> (1994)      |
| $\lambda$ foo            | Vector for fusion to C terminus of gpV                    | Maruyama <i>et al.</i> (1994)      |
| $\lambda$ fooDn          | Vector for fusion to N terminus of gpD                    | This work                          |
| $\lambda$ fooDc          | Vector for fusion to C terminus of gpD                    | This work                          |
| $\lambda$ PAn            | $\phi$ (protein A-gpD)                                    | This work                          |
| $\lambda$ PAC            | $\phi$ (gpD-protein A)                                    | This work                          |
| $\lambda$ D $\beta$ gal  | $\phi$ (gpD- $\beta$ -galactosidase)                      | This work                          |
| <b>C. <i>E. coli</i></b> |   |                                    |
| EQ166                    | <i>sup</i> <sup>0</sup> , a suppressor-negative strain    | Maruyama <i>et al.</i> (1994)      |
| MC8                      | <i>supG</i>   | Maruyama <i>et al.</i> (1994)      |
| Q358                     | <i>supE</i>   | Maruyama <i>et al.</i> (1994)      |
| TG1                      | <i>supE</i>   | Maruyama <i>et al.</i> (1994)      |
| WXO                      | <i>supF</i>   | S. Brenner                         |

\* Multiple cloning sites.

<sup>b</sup>  $\phi$ (A-B) indicates fusion of A to the N terminus of B.

We constructed also plasmid vectors for fusion to the C terminus of gpD. PCR reaction was designed to introduce *Nhe*I and *Eag*I restriction sites immediately after the termination codon of the *D* gene, resulting in p $\lambda$ Dne (Figure 1). This PCR mutagenesis also replaced the original ochre stop

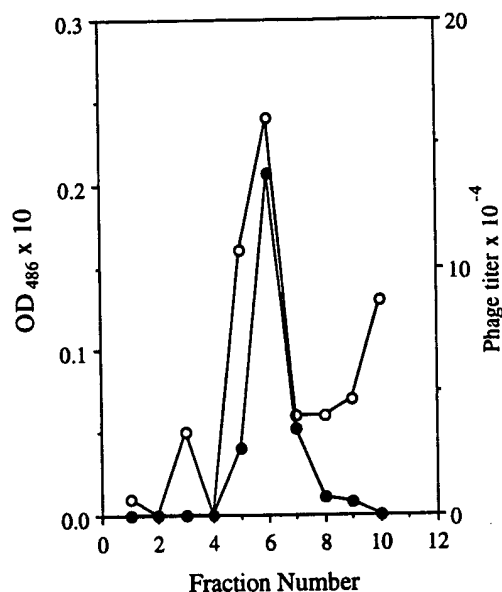
codon with an amber codon for the conditional fusion through a host suppressor activity. From p $\lambda$ Dne, p $\lambda$ D3 was created through p $\lambda$ D by inserting a PCR fragment encoding a collagenase cleavage site, proline and threonine repeats, multiple cloning sites, and *lacZ'*. In order to test the vector p $\lambda$ D3 for

Table 2. EDTA sensitivity of  $\lambda$ F7 phage with wild-type or modified gpD

| <i>E. coli</i> host               | Plasmid*       | Fusion product         | Recovery<br>(% of input phages <sup>b</sup> ) |
|-----------------------------------|----------------|------------------------|---|
| EQ166 ( <i>sup</i> <sup>0</sup> ) | —              | —                      | 0.0   |
| Q358 ( <i>supE</i> )              | —              | —                      | 116.7   |
| EQ166 ( <i>sup</i> <sup>0</sup> ) | p $\lambda$ D  | gpD                    | 91.3  |
| EQ166 ( <i>sup</i> <sup>0</sup> ) | p $\lambda$ D1 | gpD                    | 7.3   |
| EQ166 ( <i>sup</i> <sup>0</sup> ) | p $\lambda$ AD | Protein A-gpD          | 27.3  |
| EQ166 ( <i>sup</i> <sup>0</sup> ) | p $\lambda$ LD | $\beta$ -Lactamase-gpD | 0.6   |

\* p $\lambda$ D and p $\lambda$ D1 encode unfused gpD; p $\lambda$ AD, fusion of IgG-binding domains to the N terminus of gpD; p $\lambda$ LD, fusion of  $\beta$ -lactamase to the N terminus of gpD. Plasmid p $\lambda$ D and p $\lambda$ AD were constructed from a high copy number vector, pBSC; p $\lambda$ D1 and p $\lambda$ LD were from a low copy number vector, pACYC184.

<sup>b</sup> Approximately 10<sup>7</sup> pfu (plaque-forming units) phages were treated with EDTA as described in Materials and Methods. Data are the mean of three independent measurements.

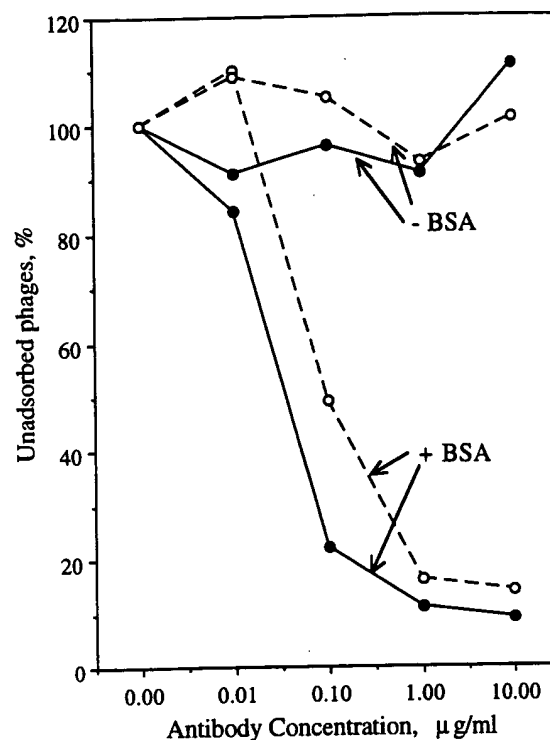


**Figure 2.** Co-migration of  $\beta$ -lactamase activity and phage particles.  $\lambda$ F7 phage lysate from EQ166 harbouring p $\lambda$ LD was fractionated through a CsCl step-gradient and each fraction was titrated for phages ( $\bullet$ ) and  $\beta$ -lactamase activity ( $\circ$ ). p $\lambda$ LD encodes the  $\beta$ -lactamase-gpD fusion product.

C-terminal fusion of gpD, a *Hind*III-*Eco*RI fragment, encoding the IgG-binding domain of *S. aureus* protein A, was excised from p $\lambda$ AD and cloned into p $\lambda$ D3 to create p $\lambda$ DA, which encodes protein A fused to the C terminus of gpD. The p $\lambda$ DA plasmid should produce both fused and unfused gpD in the presence of an amber suppressor of *E. coli* hosts such as Q358 and TG1.  $\lambda$ F7 and  $\lambda$ 2001 phages grown on TG1 containing p $\lambda$ DA bound to anti-BSA antibody, and were adsorbed to a microtiter plate coated with BSA (Table 3). This suggested that the fusion product was incorporated into  $\lambda$ F7 and  $\lambda$ 2001 phage heads together with unfused gpD.

### Recovery of phage bound to solid matrices

The plasmid fusion vectors described above were designed to encode endopeptidase cleavage sites at their fusion junction (Figure 1(b) and (c)) for the efficient recovery of bound phages from solid matrices under mild conditions.  $\lambda$ F7 or  $\lambda$ 2001 grown on EQ166 harbouring p $\lambda$ AD or p $\lambda$ DA was adsorbed to a BSA-coated microtiter plate after mixing with anti-BSA antibody and bound phages were recovered as infectious phages from the plate by collagenase digestion (Table 3). Higher recovery of phages carrying both fused and unfused gpD ( $\lambda$ 2001, and  $\lambda$ F7 grown in TG1 harbouring p $\lambda$ DA) was observed than that of phage carrying only fusion gpD ( $\lambda$ F7 grown on EQ166 harbouring p $\lambda$ AD). This is reasonable because phages with more fusion molecules may be released less efficiently by collagenase digestion from the plate. We also tried to recover bound phages by the



**Figure 3.** Adsorption of fusion phages to a microtiter plate. The IgG-binding activity of protein A domains fused to the phage particles was examined by its ability to bind to anti-BSA antibody.  $\lambda$ F7 phage ( $\circ$ ) and  $\lambda$ 2001 phage ( $\bullet$ ) lysates prepared from EQ166 harbouring p $\lambda$ AD were incubated with anti-BSA antibody and were applied to a microtiter plate coated with BSA. After incubation, the supernatant was titrated for unadsorbed phages.

addition of bacteria directly to the wells for infection and found that this method was constantly ten times less efficient than collagenase extraction (data not shown). The p $\lambda$ AD phage also contains the recognition sequence for the factor Xa endopeptidase (Figure 1(b)) but this enzyme failed to release phage from the plate. However, phages treated with factor Xa prior to incubation with

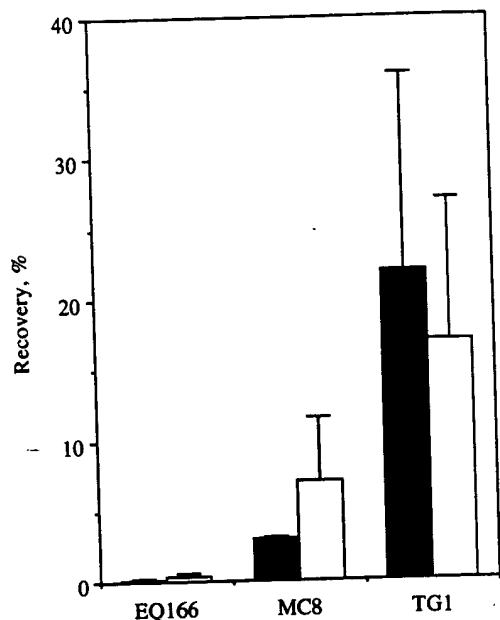
**Table 3.** Phage elution by collagenase

| <i>E. coli</i> host               | Plasmid*       | Antibody | Recovery <sup>b</sup> (pfu) |                |
|-----------------------------------|----------------|----------|-----------------------------|----------------|
|                                   |                |          | $\lambda$ F7                | $\lambda$ 2001 |
| EQ166 ( <i>sup</i> <sup>0</sup> ) | p $\lambda$ AD | +        | 2400                        | 17,000         |
| EQ166 ( <i>sup</i> <sup>0</sup> ) | p $\lambda$ AD | -        | 0                           | 0              |
| TG1 ( <i>sup</i> E)               | p $\lambda$ DA | +        | 40,000                      | 8000           |
| TG1 ( <i>sup</i> E)               | p $\lambda$ DA | -        | 0                           | 0              |

The  $2 \times 10^7$  pfu phages (100  $\mu$ l) were mixed with or without 1.0  $\mu$ l of anti-BSA antibody (0.22  $\mu\text{g}/\mu\text{l}$ ) and were bound to microtiter wells coated with BSA. Phage bound to the wells were eluted by collagenase treatment as described in Materials and Methods, and titrated on WXO.

\* p $\lambda$ DA produces both unfused gpD and fused gpD to the N terminus of the IgG-binding domain of protein A, and p $\lambda$ AD produces only a fusion product of the domains fused to the N terminus of gpD since it has no amber stop codon at the fusion junction.

<sup>b</sup> Data are the mean of three independent measurements.

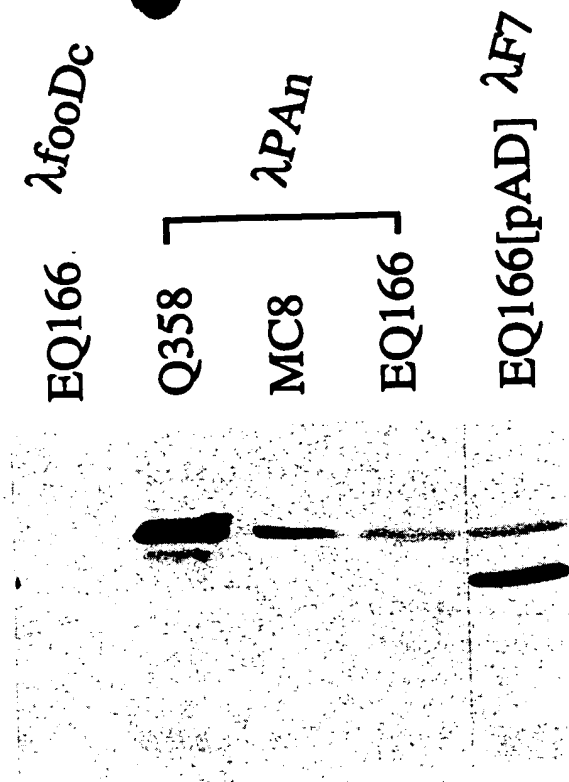


**Figure 4.** Binding of phages grown on bacteria with various suppressor activities.  $\lambda$ PAn (filled bars) and  $\lambda$ Pac (open bars) phages were grown on the bacteria indicated and partially purified by PEG precipitation. Phages, approximately  $1.0 \times 10^7$  pfu, were applied to microtiter wells coated with rabbit anti-BSA antibody. Bound phages were eluted by collagenase treatment and titrated on WKO. The data are the mean  $\pm$  SD of three separate measurements.

antibody were less efficiently adsorbed (2.7%) to the plate than those untreated (38%). This suggested that the factor Xa sequence could serve as an enzyme cleavage site between protein A and gpD but could not be accessed by the enzyme in the phage-protein A-antibody complex attached to the plate, probably due to steric hindrance.

### Phage vectors

$\lambda$  phage vectors should be more suitable than plasmid vectors for construction of complex libraries such as cDNA libraries. We constructed also two phage vectors,  $\lambda$ fooDn and  $\lambda$ fooDc, by replacing the *Apa*I-*Apa*LI fragment of  $\lambda$ foo with the modified *D* genes on p $\lambda$ D $\alpha$  and p $\lambda$ D $\beta$ , respectively (Figure 1). p $\lambda$ D $\alpha$  and p $\lambda$ D $\beta$  were derived from p $\lambda$ Da and p $\lambda$ Dne, respectively, by inserting a PCR product encoding proline and threonine repeats of  $\lambda$ foo (for details, refer to Materials and Methods). To examine the incorporation of fusion proteins encoded by the phage vectors into their heads, the IgG-binding domain of *S. aureus* protein A was fused to the N terminus of gpD using  $\lambda$ fooDn and to the C terminus of gpD using  $\lambda$ fooDc, resulting in  $\lambda$ PAn and  $\lambda$ Pac, respectively. These phages were grown in bacteria having various suppressor activities and the IgG-binding activity of the phages was verified by adsorption of the phages pre-mixed with anti-BSA antibody to microtiter wells coated with BSA. As shown in Figure 4, the binding of the



**Figure 5.** Western blot of the gpD product incorporated into phage particles.  $\lambda$ PAn were grown on *E. coli* strains EQ166, MC8 or Q358, which have no, weak or strong suppressor activity, respectively, and partially purified by precipitation with PEG. Phage proteins were separated by gel electrophoresis, blotted onto a nitrocellulose filter, and stained with anti-BSA antibody as described in Materials and Methods. As references,  $\lambda$ fooDc vector without an insert (left) and  $\lambda$ F7 grown on EQ166 harbouring pAD (right) are shown. The plasmid pAD encodes the IgG-binding domain B fused to the N terminus of gpD. The bands on the lanes of  $\lambda$ PAn grown on Q358 and MC8 correspond to a protein A IgG-binding domain B-gpD fusion product with a molecular mass of 33.0 kDa; the difference between the molecular masses of the fusion proteins encoded by  $\lambda$ PAn and pAD is due to the linker sequences on  $\lambda$ PAn. A lower band on the  $\lambda$ F7 lane corresponds to protein A IgG-binding domain B fused to the N terminus of gpD, whose molecular mass is 30.6 kDa, close to the estimated value of 29.2 kDa. The faint band on the lanes of  $\lambda$ F7 and  $\lambda$ PAn grown on EQ166 is an unknown protein that is co-purified with protein A and cross-reacts with either primary or secondary antibody.

fusion phage was proportional to the suppressor activity of *E. coli* hosts used for the phage culture. When a strong suppressor strain, such as TG1, was used for phage culture, binding of the phage to the plate was more efficient than that of phages grown on a weaker strain, such as MC8. These results indicated that the IgG-binding domain of protein A was successfully displayed with IgG-binding activity on the surface of the phage particle. The fusion was further substantiated by western blots of purified phage particles, which were probed with anti-BSA antibody (Figure 5). Fusion proteins

Table 4. Affinity selection of  $\lambda$ PAn and  $\lambda$ PAC fusion phages

| Input         |                    | Output        |                    | Yield (%) <sup>a</sup> | Enrichment factor <sup>b</sup> |
|---------------|--------------------|---------------|--------------------|------------------------|--------------------------------|
| $\lambda$ PAC | $\lambda\beta$ gal | $\lambda$ PAC | $\lambda\beta$ gal |                        |                                |
| 270           | $1.1 \times 10^8$  | 275           | 150                | 102                    | $7.5 \times 10^5$              |
| 2700          | $9.3 \times 10^7$  | 1161          | 240                | 43                     | $1.7 \times 10^5$              |
| 270           | $9.7 \times 10^6$  | 54            | 43                 | 20                     | $4.5 \times 10^4$              |
| 2700          | $8.7 \times 10^6$  | 864           | 63                 | 32                     | $4.4 \times 10^4$              |
| $\lambda$ PAn | $\lambda\beta$ gal | $\lambda$ PAn | $\lambda\beta$ gal |                        |                                |
| 420           | $9.3 \times 10^7$  | 378           | 75                 | 90                     | $1.1 \times 10^6$              |
| 4200          | $9.3 \times 10^7$  | 4116          | 75                 | 98                     | $1.2 \times 10^6$              |
| 420           | $7.5 \times 10^6$  | 357           | 34                 | 85                     | $1.9 \times 10^5$              |
| 4200          | $8.2 \times 10^6$  | 3738          | 38                 | 89                     | $1.9 \times 10^5$              |

$\lambda$ PAn and  $\lambda$ PAC phages grown on Q358 were respectively mixed with  $\lambda\beta$ gal in various ratios indicated and were selected through a single round of affinity selection using a microtiter plate coated with rabbit anti-BSA antibody. Extracted phages with collagenase were titrated on EQ166 in the presence of X-gal. In this experiment,  $\lambda\beta$ gal was used as an internal reference, since it formed blue plaques and could be distinguished from white  $\lambda$ PAC or  $\lambda$ PAn plaques. Data are the mean of three independent measurements.

<sup>a</sup> Yields of  $\lambda$ PAC and  $\lambda$ PAn are as the ratio of output to input.

<sup>b</sup> Enrichment factors are calculated by the ratio of output ratio,  $\lambda$ PAC/ $\lambda\beta$ gal, to input ratio,  $\lambda$ PAC/ $\lambda\beta$ gal.

migrated as a band having molecular mass of 33.0 kDa, which was close to the calculated molecular mass of 32.0 kDa. The amount of fused protein A to phage capsids depended on the *E. coli* strains used for phage growth, consistent with the results that binding activity of fusion phages was highly dependent on suppressor activity of bacterial hosts.

Using the  $\lambda$ PAn and  $\lambda$ PAC phage constructs, an affinity selection experiment was carried out to examine how efficiently the phages were purified over a reference phage,  $\lambda\beta$ -gal (Table 4). Both the phages  $\lambda$ PAn and  $\lambda$ PAC were very efficiently purified with antibody immobilized on a microtiter plate by a single round of affinity selection. While the enrichment factor was equivalent to that obtained with  $\lambda$ foo fusion phages (Maruyama *et al.*, 1994), recovery yields of the phages were 10 to 100 times better than those obtained with  $\lambda$ foo fusion phages. These very high yields suggested that a small number, 100 or less pfu, of phage of interest could be affinity-purified from libraries containing a very large number of variants, such as a cDNA library or random peptide library.

In order to estimate the number of foreign molecules displayed on the phage surface, we fused  $\beta$ -galactosidase to the C terminus of gpD using  $\lambda$ fooDc and termed the construct  $\lambda$ D $\beta$ -gal. A similar construct using  $\lambda$ foo,  $\lambda\beta$ -gal, was analysed by electron microscopy to quantify the number of  $\beta$ -galactosidase molecules and the result indicated ~0.5 molecule of the enzyme, on average, per phage particle (Maruyama *et al.*, 1994). Therefore, by direct comparison of the  $\beta$ -galactosidase activity of purified  $\lambda$ D $\beta$ -gal particles to that of  $\lambda\beta$ -gal, the mean number of the enzyme molecules incorpor-

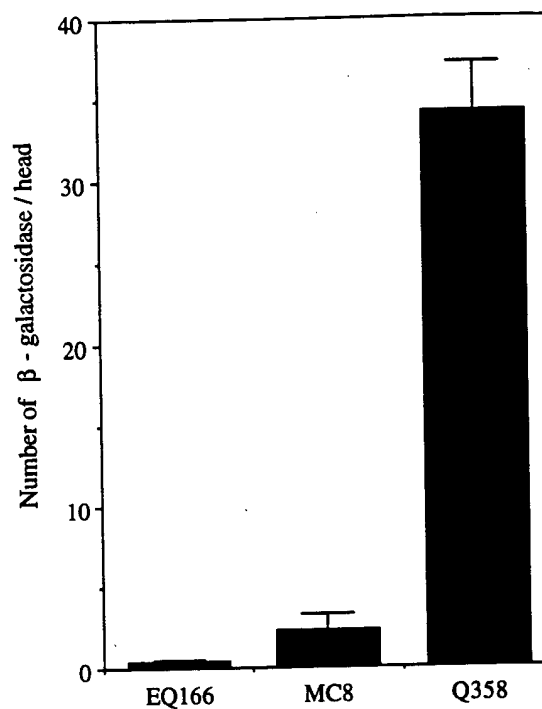


Figure 6. Estimated number of  $\beta$ -galactosidase molecules per phage particle. The numbers are estimated from relative enzyme activity of purified  $\lambda$ D $\beta$ -gal phage particles, grown on the *E. coli* host indicated, to that of  $\lambda\beta$ -gal phage grown on MC8. Data are the mean  $\pm$  SD of three separate preparations.

ated into a phage capsid was estimated for  $\lambda$ fooDc. As shown in Figure 6, the mean number of  $\beta$ -galactosidase molecules displayed on a phage capsid was 34.3 for  $\lambda$ D $\beta$ -gal grown on Q358, which has a strong suppressor activity, while 2.3 for  $\lambda$ D $\beta$ -gal grown on MC8, which has a weak suppressor activity. These results suggested that the incorporation of  $\beta$ -galactosidase molecules fused to gpD into phage heads was more efficient than that obtained with gpV fusion into phage tails.

## Discussion

We have constructed vectors that allow fusion of proteins to either the N or C terminus of the D protein (gpD) of bacteriophage  $\lambda$ , permitting efficient display on the surface of the phage head. This shows that both ends of the gpD polypeptide chain are exposed on the external surface of the trimer. The ends are neither at the trimer interaction face nor at the surface that interacts with the E protein, the major head protein. This is an important result for which no guarantee existed at the start of this work.

Most phage display systems involve fusion at the N terminus of phage proteins although C-terminal fusions have been made for the V gene (Maruyama *et al.*, 1994; Dunn, 1995) and filamentous phage protein VI (Jespersen *et al.*, 1995). For C-terminal fusions with gpD, we have employed the same linker as previously used with  $\lambda$ foo. This encodes



an endopeptidase cleavage site, long proline-threonine repeats, a ribosome-binding site, and a methionine initiator codon. Therefore, the vector expresses both fused and unfused gpD in various ratios depending on the suppressor tRNA activity of *E. coli* hosts to be used. The linker of proline-threonine repeats is unlikely to interfere with independent folding of the two distinct domains, gpD and foreign proteins. The endopeptidase recognition sequences between the two domains can be enzymatically cleaved, allowing infectious phage particles to be released from solid matrices under mild conditions.

Both of the plasmid vectors (p $\lambda$ D5, p $\lambda$ D3) and phage vectors ( $\lambda$ fooDn,  $\lambda$ fooDc) should be useful for the display of peptides or proteins. However, the C-terminal fusion vectors (p $\lambda$ D3 and  $\lambda$ fooDc) may be more suitable for expression of cDNA because of the existence of many stop codons in the 3' untranslated region. For the expression of DNA-binding proteins, the N-terminal fusion vectors (p $\lambda$ D5 and  $\lambda$ fooDn) may be more appropriate since many DNA-binding proteins such as Fos or lambda repressor have the binding sites at their N-terminal regions and the N-terminal fusion vector fuses the C terminus of the DNA-binding domain to the N terminus of gpD. Sternberg and Hoess's N-terminal D fusion display phage system requires the *loxP*-*cre* site-specific recombination-mediated incorporation from plasmid library and lambda transduction steps (Sternberg & Hoess, 1995). The phage vectors  $\lambda$ fooDn and  $\lambda$ fooDc described here can be used directly to construct large libraries of variant peptides and proteins. Using these vectors, the ratio of fused gpD molecules to unfused on the phage head can be controlled by the suppressor tRNA activity of *E. coli* hosts. This is an important feature for the expression of foreign proteins that may disturb the phage assembly. Without this feature, such clones might be under-represented in libraries.

EDTA-resistant phage could be recovered from  $\lambda$ F7 phage grown on bacteria producing only completely fused gpD (Table 2), suggesting that almost the maximum number of molecules, ~400, of a fusion gpD can be incorporated into the phage particle. This high level of incorporation could be useful for affinity purification of particular clones from a library and for other experiments, such as raising antibodies against the displayed protein (Willis *et al.*, 1993) and gene delivery (Hart *et al.*, 1994).

## Materials and Methods

### Construction of D fusion vectors

In order to modify the 5' end of the *D* gene, a pair of oligonucleotide primers, 5'-GGATCCGGGGGTATTAATATGACGGGTACCAGCAAAGAAACCTTTACC and 5'-ATCGGCCGGTTCGACTTAAACGATGCTGATTGC, were used for PCR amplification of the gene (Saiki *et al.*, 1988) using  $\lambda$ 1685 DNA as a template. The former primer contains the ribosome-binding site of the *S. aureus* protein A gene (Uhlén *et al.*, 1984). The PCR fragment digested

with *Bam*HI and *Eag*I was cloned between *Bam*HI and *Eag*I of pACYC184, resulting in p $\lambda$ D1.

Separately from the above construction, a 4587 bp *Bam*HI-*Apa*I fragment of  $\lambda$ 1685 DNA encompassing the genes 'C', 'Nu3', *D*, *E*, *F*I, *F*II, *Z*, *U*, *V* and *G*' was also cloned into pUM15 for the mutagenesis of the *D* gene, resulting in p $\lambda$ Do. The pUM15 plasmid is a derivative of pUM13 (Maruyama & Brenner, 1992) and has *Apa*I and *Bam*HI sites in the multiple cloning site (MCS). In order to create a unique *Sfi*I site between the second and third codons of the *D* gene, two pairs of oligonucleotide primers, 5'-CCGGGGATCCTCAACTGTGAGGA and 5'-ATGGCCCCGGGGGCCGTCATAAACATCCCTTACACTG, and 5'-TTGGCCCCGGGGGCCAGCAAGAAACCTTTACCCATTA and 5'-TGCCCTTAAGCAGGCGAGAAACT, were used for PCR amplification of the gene. To create p $\lambda$ Ds, the two PCR fragments digested with *Bam*HI and *Sfi*I, and *Sfi*I and *Afl*II, respectively, were cloned in tandem between the *Bam*HI and *Afl*II sites of p $\lambda$ Do. After eliminating all the remaining MCS of pUM15, a new MCS cassette encoding *Hind*III, *Sph*I, *Pst*I, *Acc*I, *Hinc*II, *Xba*I, *Bam*HI, *Sma*I, *Kpn*I, *Sac*I, *Eco*RI and *Hae*III was introduced into the unique *Sfi*I site located at the 5' end of the *D* gene on p $\lambda$ Ds, resulting in p $\lambda$ Da. A synthetic cassette encoding a collagenase recognition sequence (Germino & Bastia, 1984), factor Xa recognition sequence (Maina *et al.*, 1988) and linker sequence was cloned into the *Kpn*I and *Sfi*I sites in p $\lambda$ Da, creating the plasmid p $\lambda$ Db. The plasmids described above were used for the fusion of  $\beta$ -lactamase or the IgG-binding domain of protein A to the N terminus of gpD. After confirming that the fusion proteins were functionally active and were incorporated into the phage particle, the p $\lambda$ D5 vector was constructed by the following procedures. The linker sequence of  $\lambda$ foo (Maruyama *et al.*, 1994) was amplified by PCR with primers, 5'-GAATTCAGCGGCCGATAGCCGACCGGGCCAAATTCTATCGAAGGTCGTGGGACTCCGACCCCGACCACTCCC and 5'-AATGGCCCCGGGGGCCGTAATCATGGTCATAGC. The former primer contains a *Not*I site, amber stop codon and endopeptidase recognition sequence. After digesting with *Eco*RI and *Sfi*I, the PCR fragment was cloned into p $\lambda$ Da to produce p $\lambda$ D $\alpha$ . A fragment containing the modified *D* gene was cut out with *Pst*I and *Pvu*II from p $\lambda$ D $\alpha$  and cloned into the *Pst*I and *Sma*I sites in pBSC to make p $\lambda$ D5.

For construction of plasmid vectors for the C-terminal fusion of gpD, *Nhe*I and *Eag*I sites were introduced between the *D* and *E* genes by PCR using two pairs of oligonucleotide primers, 5'-CCGGGGATCCTCAACTGTGAGGA and 5'-AACGGCCGAATGCTAGCGATAACGATGCTGATTGCCGTTCGGGC, and 5'-AACGGCCGCTTTACCCCTTCATCACTAAAG and 5'-TGCCCTTAGCAGCGCAGAAACT, and  $\lambda$ 1685 as a template. The two PCR fragments were digested with *Bam*HI and *Eag*I, and *Eag*I and *Afl*II, respectively, and were cloned in tandem between the *Bam*HI and *Afl*II sites in p $\lambda$ Do to create p $\lambda$ Dne. The *D* gene ochre termination codon, TAA, was replaced with an amber termination codon, TAG, in p $\lambda$ Dne. DNA encoding a collagenase recognition sequence, peptide linker, MCS and *lacZ'* was amplified by PCR from  $\lambda$ foo DNA, using primers, 5'-AAGCTAGCAGCTGGCCTGTGGGCCCCACT and 5'-AACGGCCGCTATCTATCTAGAATTCGAGCTCGGTACCCGGG. This PCR fragment was digested with *Nhe*I and *Eag*I, and cloned between the *Nhe*I and *Eag*I sites in p $\lambda$ Dne, resulting in plasmid p $\lambda$ Db. A *D* gene fragment was also isolated from p $\lambda$ Dne with *Bam*HI and *Eag*I and cloned into *Bam*HI and *Eag*I in pBSC. By eliminating MCS

between *Kpn*I and *Bam*HI in pBSC, p $\lambda$ D was created. The PCR fragment encoding a collagenase recognition sequence, peptide linker, MCS and *lacZ'* was inserted into *Nhe*I and *Eag*I in p $\lambda$ D to make p $\lambda$ D3. All oligonucleotide primers used in this work were synthesized using an Applied Biosystem DNA synthesizer model 394 (Foster City, CA). Sequences of all PCR products were confirmed by DNA sequencing (Sanger *et al.*, 1977), since PCR induces mutations at a high rate (Maruyama, 1990; Williams & Winter, 1993).

A 4755 bp *Apa*I-*Apa*LI fragment of p $\lambda$ D $\alpha$  and a 4950 bp *Apa*I-*Apa*LI fragment of p $\lambda$ D $\beta$  were cut out with *Apa*I and *Apa*LI, and ligated with left and right arms purified from  $\lambda$ foo DNA digested with *Apa*I and *Apa*LI to make phage vectors,  $\lambda$ fooDn and  $\lambda$ fooDc, respectively. *In vitro* packaging of the ligated DNAs was carried out as described (Maruyama *et al.*, 1994).

### Cloning of genes into vectors

The  $\beta$ -lactamase gene was isolated from pUC18 by PCR amplification, using primers 5'-GAGGTACCCTGGTGAAGTAA and 5'-GTGGTACCCTCGAGCCAAATGCTTAATCAGTGA. The amplified DNA was digested with *Kpn*I and cloned into the *Kpn*I site of p $\lambda$ D1, creating p $\lambda$ LD.

Plasmid pRIT5 was used as a template for PCR to amplify a DNA fragment encoding the IgG-binding domains of protein A, using primers 5'-TTCTGCAGCGCGCAACACGATGAAGCTCAA and 5'-TTGGTACCGCTCACCGAAGGATCGTC. The PCR fragment encoding the domains E, D, A and B was digested with *Pst*I and *Kpn*I, and inserted into the *Pst*I and *Kpn*I sites of p $\lambda$ Db to fuse the domains to the N terminus of gpD. To produce the protein A-gpD fusion protein in bacteria, the fragment encoding the protein A-gpD fusion was cut out with *Pst*I and *Pvu*II, and cloned into the *Pst*I and *Sma*I sites of pBSC. The resulting construct was designated p $\lambda$ AD, in which expression of the fusion protein was placed under the control of the *lac* promoter. A DNA fragment encoding the B domain of protein A was cut out with *Hind*III and *Eco*RI from p $\lambda$ AD and cloned into the *Hind*III and *Eco*RI sites of p $\lambda$ D3, resulting in plasmid p $\lambda$ DA. p $\lambda$ DA, a deletion derivative of p $\lambda$ AD, was made by eliminating a 465 bp *Hind*III fragment encoding the IgG-binding domains E, D and A of protein A.

For the construction of  $\lambda$ PAn, the protein A gene was isolated from p $\lambda$ AD by digestion with *Hind*III and *Eco*RI, and cloned into the *Hind*III and *Eco*RI sites of  $\lambda$ fooDn. Similarly,  $\lambda$ PAC was constructed from a *Hind*III-*Eco*RI fragment of p $\lambda$ AD and  $\lambda$ fooDc digested with *Hind*III and *Eco*RI. The  $\beta$ -galactosidase gene was excised from pMC1871 by *Bam*HI and cloned into  $\lambda$ fooDc to make  $\lambda$ D $\beta$ -gal.

### Fusion protein expression

gpD and its fusion products were produced intracellularly at a higher level from pBSC-based vectors (p $\lambda$ D, p $\lambda$ AD and p $\lambda$ DA) or at a lower level from pACYC184-based vectors (p $\lambda$ D1 and p $\lambda$ LD). *E. coli* strains harbouring plasmid p $\lambda$ D, p $\lambda$ AD or p $\lambda$ DA were grown in CY medium (Maruyama *et al.*, 1994) containing 10 mM MgSO<sub>4</sub> and 100  $\mu$ g/ml ampicillin, and *E. coli* strains harbouring plasmid p $\lambda$ D1 or p $\lambda$ LD were grown at 37°C overnight in the same medium containing 10  $\mu$ g/ml chloramphenicol.  $\lambda$ F7 was added to a 1.0 ml overnight culture of bacteria at 0.1 multiplicity of infection. After adsorption at 37°C

for ten minutes, the culture was diluted 100-fold with CY containing 10 mM MgSO<sub>4</sub> and appropriate antibiotics, and incubated with vigorous agitation at 37°C until complete cell lysis. When TG1 was used as a host, culture was diluted with CY containing 10 mM MgSO<sub>4</sub>, 100  $\mu$ g/ml ampicillin, and 1.0 mM isopropyl- $\beta$ -D-thiogalactopyranoside.

### Assay of fusion proteins

For the EDTA-sensitivity test, phages were incubated either with TM buffer (10 mM Tris-HCl (pH 7.4), 10 mM MgSO<sub>4</sub>) or TE buffer (10 mM Tris-HCl (pH 7.4), 10 mM EDTA) for 15 minutes at 37°C and titrated on *E. coli* strain WXO, as described by Sternberg & Weisberg (1977).

The activity of  $\beta$ -lactamase displayed on phage heads was assayed with Nitrocefin (Becton Dickinson, Cockeysville, MD) as a colour indicator (O'Callaghan *et al.*, 1972). For phage purification, CsCl step-gradients were prepared: first layer, 5.95 g CsCl in 3.5 ml of TM buffer; second layer, 3.75 g CsCl in 2.5 ml of TM buffer; third layer, 3.25 g CsCl in 2.5 ml of TM buffer.  $\lambda$ F7 phage lysate grown on *E. coli* EQ166 containing p $\lambda$ LD was fractionated through the CsCl step-gradient by centrifugation at 15,000 g for 2.4 hours at 4°C. Ten-drop samples were collected from the bottom of the tube. The phage titer of each fraction was measured after incubating in TE buffer for 15 minutes at 37°C, using WXO as a host.  $\beta$ -Lactamase activity was measured as absorbance at 486 nm after the addition of Nitrocefin to the fractions.

The IgG-binding activity of protein A fused to phage heads was tested by its ability to bind to rabbit anti-BSA antibody (Sigma, St Louis, MO). Microtiter wells were coated with 1 mg/ml BSA in PBS (130 mM NaCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, (pH 7.2)) at 4°C for 12 to 16 hours. The wells were rinsed three times with distilled water, filled with blocking buffer (PBS, 0.05% (v/v) Tween-20, 5% (w/v) skimmed milk, 0.5% (w/v) gelatin) at room temperature for 30 minutes, and then rinsed three times with distilled water. Phage was mixed with antibody in binding buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween-20, 10 mM MgSO<sub>4</sub>) at 4°C for 12 to 16 hours and applied to the plate. After two hours of incubation at room temperature, a 1  $\mu$ l aliquot was removed, diluted with  $\lambda$ -dil (Maruyama *et al.*, 1994), and titrated on WXO for infectious phages. Phage adsorbed to the plate was also recovered by collagenase (Sigma) treatment as described by Maruyama *et al.* (1994), and a 1  $\mu$ l aliquot was also taken from the plate and titrated on WXO. In the experiment of affinity selection of  $\lambda$ PAC and  $\lambda$ PAn, phage in  $\lambda$ -dil was applied to microtiter wells coated with rabbit anti-BSA antibody (0.22  $\mu$ g/ $\mu$ l in PBS) at 4°C overnight. Conditions for washing of the plate and elution of phage were as described above.

Assay of  $\beta$ -galactosidase activity was conducted as described by Maruyama *et al.*, (1994).

### Western blotting

$\lambda$ PAn grown in 10 ml each of EQ166, MC8 or Q358 were precipitated by the addition of PEG8000 (Fisher) to 10% (w/v) and NaCl to 1.0 M. Pellets were resuspended in 1.0 ml of  $\lambda$ -dil and reprecipitated with PEG as above. After dialysis of the phage suspension against TM, an aliquot of approximately 10<sup>8</sup> phage particles was boiled in sample buffer (62.5 mM Tris-Cl (pH 6.8), 10% (v/v) glycerol, 2.0% (w/v) SDS, 1.0% (v/v)  $\beta$ -mercaptoethanol,

0.005% (w/v) bromophenol blue) and applied to an SDS/12% polyacrylamide gel. Proteins separated by the electrophoresis were electro-transferred to a nitrocellulose filter as described by Maruyama *et al.* (1994). Protein A fused to gpD was detected by staining the blot with rabbit anti-BSA primary antibody, alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody, and BCIP/NBT (BIO-RAD) as chromogenic substrates.

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